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No evidence for an independent role of anti-heparan sulphate reactivity apart from anti-DNA in lupus nephritis

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SUMMARY

The presence of anti-heparan sulphate (HS) reactivity in serum is closely related to the occurrence of nephritis in patients with systemic lupus erythematosus (SLE). Since patients with lupus nephritis in general also have high titres of anti-DNA antibodies, we wanted to clarify the relationship between anti-HS and anti-DNA reactivity in serum. Therefore, we studied longitudinally six patients with lupus nephritis who experienced 12 exacerbations of their disease, and five SLE patients without nephritis experiencing 10 periods of non-renal disease exacerbations. In addition, we tested single serum samples of another 24 patients obtained during a renal disease exacerbation and 22 sera of patients without nephritis. The sera of all patients were tested for anti-DNA (Farr assay) and anti-HS reactivity (ELISA). We confirmed that SLE patients during renal exacerbations have a significantly higher anti-HS reactivity than patients without nephritis ($P < 0{\cdot}003$). In addition, patients with nephritis also had higher titres of anti-DNA antibodies during renal exacerbations than during non-renal exacerbations ($P < 0{\cdot}01$). A correlation between anti-DNA and anti-HS reactivity was observed ($r = 0{\cdot}40$, $P < 0{\cdot}02$), which in itself explains the correlation between nephritis and anti-HS reactivity. Comparing sera from nephritis and non-nephritis patients matched for anti-DNA titre, we found no difference in anti-HS reactivity, and therefore must conclude that the anti-HS reactivity is a direct reflection of anti-DNA reactivity.

Keywords anti-heparan sulphate anti-DNA lupus nephritis

INTRODUCTION

Systemic lupus erythematosus (SLE) is a disease of unknown etiology in which patients present a broad spectrum of autoantibodies, especially directed against nuclear constituents like DNA [1]. Disease manifestations are generally believed to be caused either by deposition or *in situ* formation of immune complexes containing these autoantigens [2–4]. An alternative explanation for the pathogenicity of anti-DNA antibodies has been the suggestion that anti-DNA antibodies directly cross-react with epitopes present within the glomerulus or glomerular basement membrane (GBM) [5–8]. However, the validity of the latter hypothesis has become a matter of debate, since lupus nephritis is strongly associated with high-avidity IgG anti-DNA antibodies, and mainly low-avidity anti-DNA antibodies do cross-react with highly negatively charged structures like cardiolipin and dextran sulphate [9,10]. Furthermore, we found that the binding of anti-nuclear antibodies to such an intrinsic glomerular antigen as heparan sulphate (HS) and to the GBM was mediated by nucleosomal antigens complexed to the anti-nuclear antibodies, and was not a feature of the antibody itself [11,12]. We therefore think that nucleosomes play an important role in the pathogenesis of lupus nephritis, moreover, since Schmiedeke and coworkers found in 1989 that histones, which constitute the nucleosome together with DNA, show a high affinity for the GBM [13]. Furthermore, nucleosomes can be detected in plasma of SLE patients [14,15]. In lupus mice, the major specificity of the initial autoantibody response is reported to be directed against nucleosomal antigens, only later in the disease to be followed by production of anti-DNA antibodies [16]. These data are in line with *in vivo* data from our group in which we found that anti-DNA antibodies can bind to the GBM after renal perfusion with histone/DNA complexes [17]. Based on these observations, we currently hold anti-DNA/nucleosome immune complexes responsible for the onset of glomerulonephritis in SLE [18,19]. Binding of anti-DNA/nucleosome immune complexes

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Table 1. Patient characteristics of the longitudinal serum study

Patient no.	Duration of period studied (months)	Number of disease exacerbations	Serum samples studied (*n*)	Disease activity during serum study
1	86	1	112	Glomerulonephritis, skin vasculitis, cerebral SLE, arthritis, thrombocytopenia
2	125	3	173	Glomerulonephritis thrombocytopenia, skin vasculitis, cerebral SLE
3	90	2	98	Glomerulonephritis leucopenia, arthralgia
4	85	2	91	Glomerulonephritis skin vasculitis
5	122	3	62	Glomerulonephritis skin vasculitis, polyarthritis
6	150	1	207	Glomerulonephritis leucocytopenia, thrombocytopenia, pericarditis
7	123	1	56	Thrombocytopenia, skin vasculitis
8	16	2	17	Pleuritis, cerebral SLE, skin vasculitis
9	135	4	177	Cerebral SLE, pleuritis, skin vasculitis, pleuropericarditis
10	113	1	135	Cerebral SLE, thrombocytopenia
11	30	2	31	Pleuritis

SLE, Systemic lupus erythematosus.

to HS in the GBM may be an important event in lupus nephritis, since HS is, as a negatively charged molecule, responsible for the charge-dependent permeability of the GBM.

Reactivity with HS *in vitro* [6,20–22] and *in vivo* [23] has been associated with human and murine lupus nephritis. In these studies it was also observed that patients with nephritis generally had higher anti-DNA titres. This prompted us to investigate the relation between anti-DNA, anti-HS and nephritis. We therefore studied longitudinally patients with lupus nephritis and patients with disease exacerbations of SLE without a sign of renal symptoms. In addition, single serum samples of patients experiencing a renal or non-renal exacerbation were tested. To elucidate the influence of anti-DNA on anti-HS reactivity we matched a number of sera of nephritis and non-nephritis patients for anti-DNA antibody level and studied the relation between anti-DNA and anti-HS in these sera.

PATIENTS AND METHODS

Patients and sera

Longitudinal study. Longitudinally obtained serum samples from 11 patients with defined SLE (fulfilling at least four of the revised American Rheumatology Association criteria [1]) were studied. The serum samples used in this study were obtained during 12 renal exacerbation periods of six patients which had a biopsy-proven diffuse proliferative SLE glomerulonephritis (WHO class IV) and during 10 periods in which five patients had non-renal manifestations without any sign of nephritis. Patient characteristics are listed in Table 1.

Cross-sectional study. Single serum samples from each of 46 patients with defined SLE were studied. Of these serum samples, 24 were obtained from nephritis patients during renal exacerbations of disease, and 22 were obtained from non-nephritis patients who experienced exacerbations of their disease.

Of all patients, 22 sera obtained during renal and 22 sera obtained during non-renal exacerbations were matched for anti-DNA reactivity, measured by Farr assay and compared with regard to their anti-HS reactivity.

All sera were stored at −20°C until use.

Anti-HS ELISA

To measure anti-HS reactivity, we used photobiotinylated HS as an antigen. The ELISA was performed as described previously [22]. Briefly, HS (Seikagaku, Tokyo, Japan) was photobiotinylated and coated overnight on streptavidin (1 μg/ml; Sigma, St Louis, MO) precoated micro-ELISA plates (Nunc, Maxisorb, Roskilde, Denmark) at 1 μg/ml. Next, the plates were washed three times with PBS containing 0·02% Tween-20 (PBS–T) and blocked with PBS containing 10% v/v normal goat serum (NGS). The coated wells were incubated with sera serially diluted in PBS–T containing 10% NGS and washed again. To assay IgG anti-HS reactivity, each well was incubated with horseradish peroxidase (HRP)-conjugated mouse MoAb to human IgG (CLB-MH-16-E, prepared in our institute) diluted 1:2000 in PBS–T. The plates were washed again and developed with 3,5,3′,5′-tetramethylbenzidine (Merck, Darmstadt, Germany) at 100 μg/ml in 0·11 M sodium acetate pH 5·5 containing 0·003% H_2O_2. On each plate serial dilutions of an anti-HS MoAb [24] as a positive control, arbitrarily set at 100 000 U/ml, and a serum of a healthy blood bank donor as a negative control were included to correct for interassay variation.

Farr assay

Sera were tested for anti-DNA reactivity using the Farr assay which was originally introduced by Wold *et al.* [25] and later modified in this institute. Details about this procedure can be found in Aarden & Smeenk [26] and Smeenk & Hylkema [27].

Statistical analysis

For statistical analysis the Mann–Whitney test was used for comparison of different groups, and Spearman's rank correlation test for paired observations. $P < 0{\cdot}05$ was considered significant.

RESULTS

Anti-DNA and anti-HS reactivity in sera of two individual patients

Longitudinally collected sera of six SLE patients with nephritis and five without nephritis were tested for anti-DNA reactivity

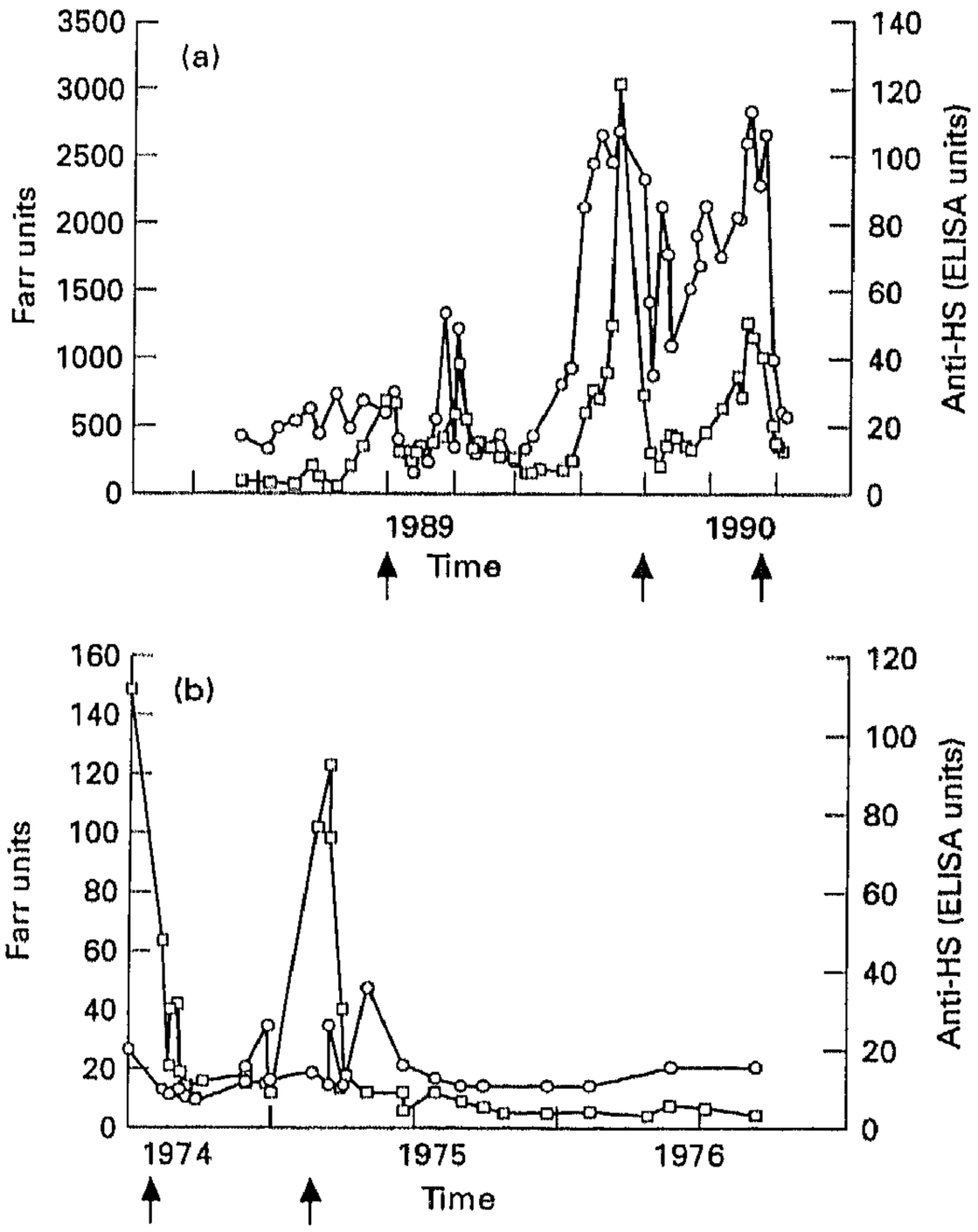

Fig. 1. Antibody profiles of a patient with (a) or without nephritis (b). The onset of disease exacerbations has been marked by an arrow. Note that the left scale has been used for the anti-DNA reactivity (□), the right scale for the anti-heparan sulphate (HS) reactivity (○).

(Farr assay) and anti-HS reactivity (ELISA). Figure 1 shows examples of the antibody pattern of an individual patient with renal exacerbations of SLE (Fig. 1a) and of a patient with exacerbations of the disease with no sign of nephritis (Fig. 1b). As can deduced from Fig. 1a, the nephritis patient experienced three periods of disease exacerbation during a period of 1 year. Besides renal manifestations, also skin, cerebral and haematological manifestations were present during the second and third exacerbation. All disease exacerbations started just after a significant peak of anti-DNA reactivity, while during remission of disease, except for one peak just after the first exacerbation, anti-DNA reactivity remained at a low level. The anti-HS reactivity pattern more or less followed the anti-DNA pattern.

The patient shown in Fig. 1b had two periods of disease exacerbation, consisting of pleuritis. Both exacerbations were preceded by a peak of anti-DNA reactivity, which remained low during remission of disease. The anti-HS pattern showed no peak reactivities during the onset of disease exacerbations.

Anti-DNA and anti-HS reactivity during renal and non-renal periods of disease exacerbation

From each exacerbation of the longitudinally studied patients, one serum sample was taken and analysed for anti-DNA and anti-HS reactivity. In this way 12 periods of renal and 10 periods of non-renal excerbations were compared.

In addition to the longitudinal study, single serum samples of 24 patients with and 22 patients without nephritis were tested for anti-DNA and anti-HS reactivity. Figure 2 summarizes the anti-DNA and anti-HS reactivities of the longitudinal and tranversal study, measured in 36 periods of renal exacerbation and 32 periods of exacerbation without nephritis. Sera of patients with nephritis had significantly higher anti-HS reactivity during periods of exacerbation than patients without nephritis ($P < 0{\cdot}003$). This phenomenon was also seen for the anti-DNA reactivity: patients with nephritis had significantly higher anti-DNA reactivity in serum than patients without nephritis ($P < 0{\cdot}01$). Furthermore, we observed that the anti-HS reactivity measured in sera of patients with or without nephritis was correlated with anti-DNA reactivity (Fig. 3, $r = 0{\cdot}40$, $P < 0{\cdot}02$). For this calculation only sera containing 20 units (95% confidence interval) or more in the anti-HS ELISA and in the Farr assay were included.

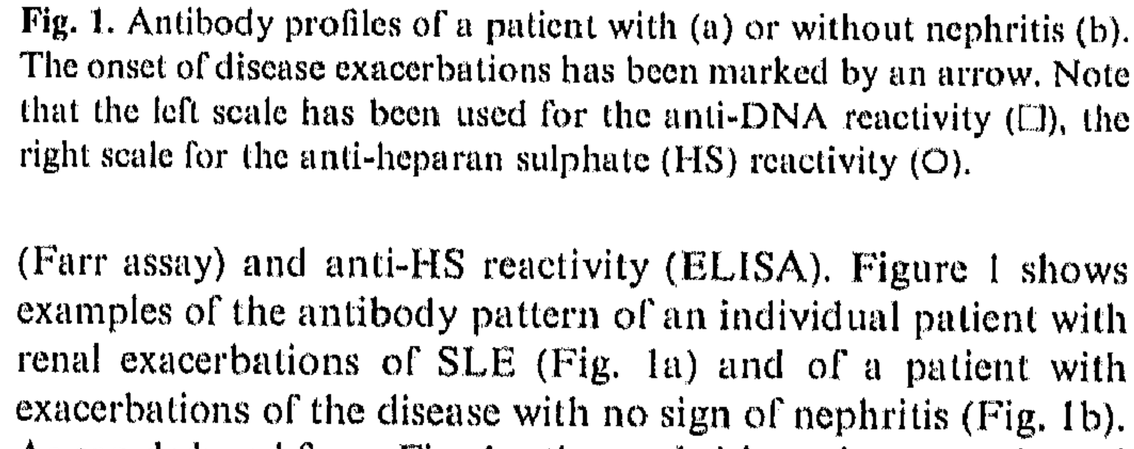

Fig. 2. Anti-heparan sulphate (HS) (a) and anti-DNA reactivity (b) of serum samples obtained from defined systemic lupus erythematosus (SLE) patients with and without nephritis, drawn during periods of disease exacerbation. Sera of patients with nephritis showed a significantly higher anti-HS ($P < 0{\cdot}003$) and anti-DNA reactivity ($P < 0{\cdot}01$) compared with sera of patients without renal exacerbations.

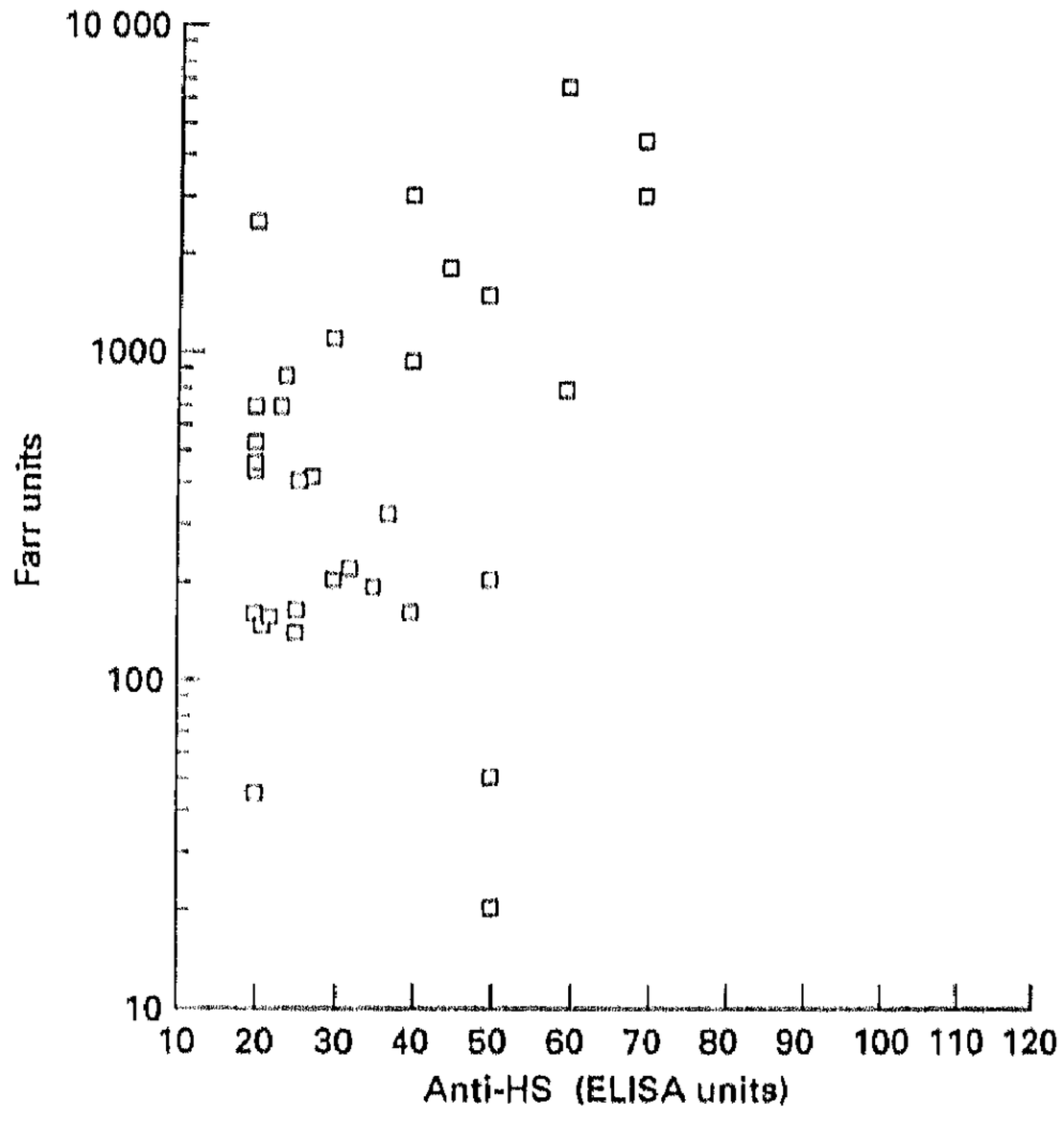

Fig. 3. Correlation between the anti-heparan sulphate (HS) and anti-DNA reactivity of serum samples which were considered positive in the anti-HS ELISA ($r = 0{\cdot}4$, $P < 0{\cdot}02$).

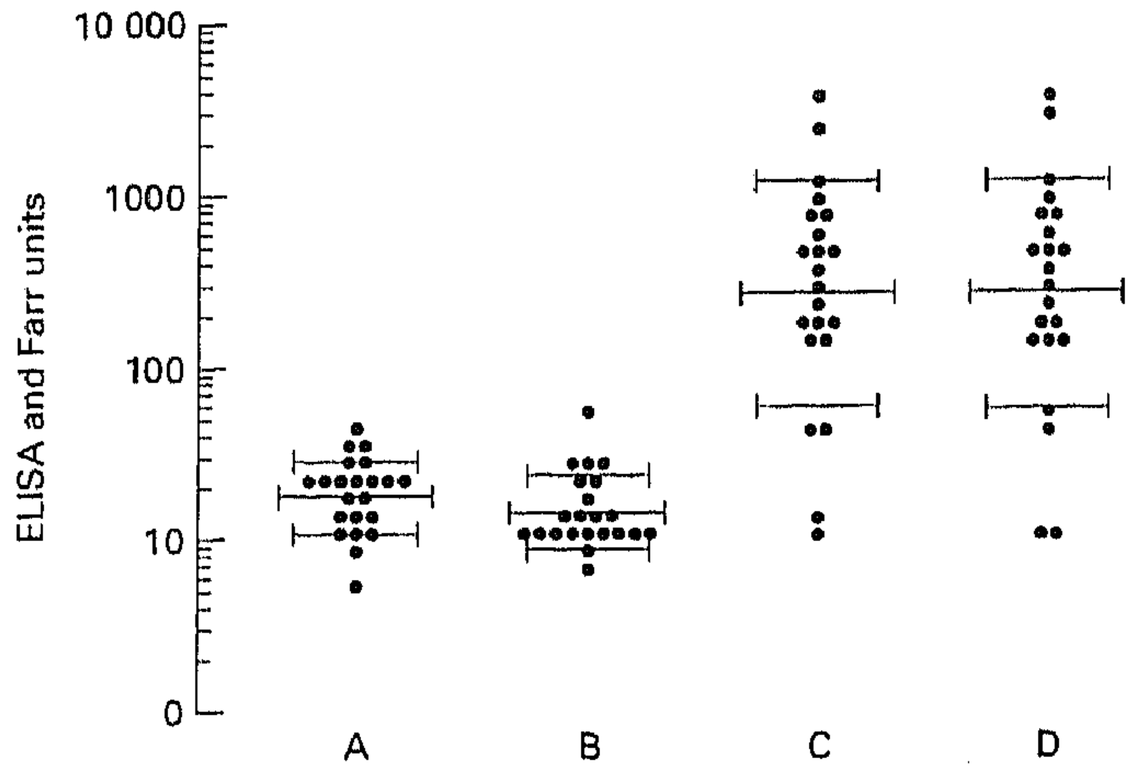

Fig. 4. Anti-heparan sulphate (HS) (A, B) and anti-DNA reactivity (C, D) during 22 renal (A, C) and 22 non-renal (B, D) exacerbations of systemic lupus erythematosus (SLE). The sera were matched for anti-DNA reactivity as measured by Farr assay. No significant difference of anti-HS reactivity could be observed between both groups of patients.

No correlation of serum anti-HS reactivity with nephritis after matching sera for anti-DNA reactivity

Analysing serum anti-HS reactivity during exacerbations, we observed a significantly increased anti-HS level during renal exacerbations in comparison with exacerbations without renal symptoms. Since the same applies to anti-DNA reactivity and since anti-DNA and anti-HS reactivity are correlated, we wanted to investigate the influence of anti-DNA on anti-HS reactivity. Therefore, of the total group of sera studied, 22 sera of nephritis patients and 22 sera of non-nephritis patients were matched for anti-DNA reactivity. For this purpose, sera with high levels of anti-DNA measured with the Farr assay had to be excluded from the nephritis patients' group because of lack of sera with similar anti-DNA reactivities in the non-nephritis group. Postulating independence of anti-DNA and anti-HS, however, one would expect this to have no influence on observed anti-HS levels. However, as depicted in Fig. 4, we did not observe a difference in anti-HS reactivity between the two groups of patients after matching for anti-DNA. Therefore, we have to reject independence of anti-DNA and anti-HS reactivities, and conclude that anti-HS is a reflection of anti-DNA reactivity. This implies that anti-HS is only correlated with nephritis indirectly, namely because of the higher anti-DNA levels found in nephritis patients.

DISCUSSION

It has always been puzzling why some patients with SLE develop glomerulonephritis whereas others do not. As anti-DNA antibodies are thought to play a role in the pathogenesis of the disease, it is not surprising that investigators tried to correlate qualitative and quantitative aspects of the anti-DNA response to the presence or absence of nephritis.

Recently, Suenaga & Abdou reported that a population of cationic high-affinity anti-DNA antibodies were present when an SLE patient had active nephritis, and that these antibodies disappeared when nephritis became inactive [28]. This study is consistent with studies done by other investigators [29,30].

Previous studies from our own laboratories demonstrated a correlation between renal symptoms during SLE disease and anti-HS reactivity, using an anti-HS ELISA with protamine chloride as an intermediate layer to obtain sufficient coating of HS to the plates [20,21]. Since that assay could be subject to false-positive results as a consequence of the binding of negatively charged immune complexes to the employed precoat (as demonstrated for the anti-DNA ELISA using the same precoat [31–33]), we developed a more anti-HS-specific ELISA, using photobiotinylated HS which can bind to streptavidin-coated wells [22]. In this ELISA, more positively charged immune complexes are measured. Furthermore, anti-HS reactivity showed a reactivity of such complexes, not of isolated antibody reacting with HS. Using this modified ELISA we also observed significantly increased anti-HS reactivity in serum obtained during renal exacerbations in comparison with sera obtained during non-renal disease manifestations. Still, not every exacerbation of SLE-nephritis was accompanied by increased levels of anti-HS reactivity. This may be explained by the possibility that free anti-DNA antibodies, that do not react with HS, localize in the glomerulus via an *in situ* immune complex mechanism once nucleosome particles have localized in the GBM. This hypothesis is in line with recent findings that some anti-DNA antibodies form circulating immune complexes, which can localize in the GBM, while others deposit via an *in situ* immune complex mechanism [17,34].

Since anti-DNA reactivity is correlated with anti-HS reactivity we wanted to elucidate the influence of anti-DNA reactivity on anti-HS reactivity. Therefore we matched from the tested panel 22 sera of patients with nephritis with 22 sera of patients without nephritis for their anti-DNA reactivity. Using this approach, no difference in HS reactivity could be found between patients with or without nephritis. This strongly suggests that anti-HS is a reflection of anti-DNA reactivity, yet theoretically the opposite, i.e. anti-DNA reactivity being a reflection of anti-HS reactivity, cannot completely be excluded. A comparable phenomenon was described several years ago by Herrera Esparza *et al.* [35] where a clearcut correlation between complement fixing anti-DNA and nephritis could only be found using sera of patients which were not matched for anti-dsDNA titre: complement fixation of anti-DNA was found to be a reflection of anti-DNA reactivity.

The observation that anti-HS reactivity seems to be mediated by anti-DNA reactivity is not very surprising, since we have already demonstrated that anti-DNA/histone/DNA complexes can bind to HS via histone molecule(s) [11,17]. Still, the Farr assay, which has been used to measure anti-DNA reactivity, has in general been considered to be highly specific for the measurement of high-avidity anti-dsDNA antibodies [10,27]. Recently, however, we showed that anti-nucleosome MoAbs, which did not bind to DNA, became Farr-positive when they were complexed to nucleosomal antigens [12]. This suggests that Farr positivity can be the result of binding of complexes composed of nucleosomal material and anti-nucleosome antibodies. The histone part of the nucleosomal particle seems to be responsible for this Farr reactivity, which is conceivable because of the DNA binding properties of this protein.

In conclusion, we have found that the correlation between anti-HS reactivity and nephritis is caused by differences in anti-DNA titre between nephritis and non-nephritis patients.

Matching sera of nephritis and non-nephritis patients for the same anti-DNA titre, we observed no difference in anti-HS reactivity between the two groups.

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